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25-Hydroxycholesterol induces mitochondria-dependent apoptosis via activation of glycogen synthase kinase-3β in PC12 cells

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Abstract
25-Hydroxycholesterol (25-OH-chol) induces apoptosis in many cell types. The present study investigated the possible involvement of mitochondria-dependent apoptotic signalling molecules in the death of PC12 cells treated with 25-OH-chol. 25-OH-chol increased the production of reactive oxygen species and opened mitochondrial permeability transition pore, resulting in release of cytochrome c and subsequent activation of caspase-9 and -3. 25-OH-chol induced the activation of c-Jun N-terminal kinase (JNK) and glycogen synthase kinase-3β (GSK-3β). The JNK inhibitor SP600125 attenuated the activation of caspase-9 and -3 and reduced 25-OH-chol-induced cell death. GSK inhibitors SB415286 and SB216763 significantly down-regulated JNK activity and attenuated the cytotoxicity of 25-hydroxycholesterol. However, SP600125 did not alter the activity of GSK-3β. The results indicate that 25-OH-chol induces cell death via activation of GSK-3β and subsequent up-regulation of JNK. Pharmacological intervention of GSK-3β-JNK-caspase signalling pathway may be useful for the reduction of cytotoxicity of oxysterols.

Keywords: *25-Hydroxycholesterol, caspase, JNK, GSK-3β*

Introduction

Oxysterols are 27-carbon products of oxidized cholesterol generated by sterol oxidation or metabolic pathways involved in the formation of sterols, steroids and bile acids. Oxysterols possess potent regulatory functions in a wide range of biological mechanisms, including cholesterol homeostasis [1], calcium uptake [2], atherosclerotic plaque formation [3] and cell differentiation [4]. Oxysterols are also cytotoxic to endothelial cells, smooth muscle cells and fibroblasts [5,6]. 25-hydroxycholesterol (25-OH-chol) is one of major cytotoxins in oxidized low density lipoprotein (oxLDL) and induces apoptosis in arterial SMC macrophages, vascular cells [7,8] and microglia and differentiated PC12 cells [9,10]. Previous studies have shown that 24OH-chol induces amyloid beta increase and promotes pro-inflammatory responses in human neuronal cell [11]. However, the exact signalling pathway underlying the cytotoxicity by 25-OH-chol remains unclear.

Mitogen-activated protein kinases (MAPKs) are serine–threonine kinases that are activated by phosphorylation in response to a wide array of extracellular stimuli [12]. Three distinct groups of MAPKs have been identified in mammalian cells (i.e. extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38). Of MAPKs, JNK has been associated with apoptosis in developing

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and mature organisms via transcription-dependent or -independent mechanisms [13,14].

Glycogen-synthase kinase-3 (GSK-3), a serine/threonine protein kinase, has been implicated in multiple biological processes including embryonic development, cell differentiation, apoptosis and insulin response [15,16]. Two isoforms of GSK-3, GSK-3α and GSK-3β, have been identified with high homology and similar but not identical biochemical properties [17]. Recent studies have indicated that GSK-3β acts upstream of mitochondria-dependent apoptosis [18]. The activity of GSK-3β is inhibited by Akt phosphorylation [19]. A role for GSK-3β in regulating apoptosis downstream of PI 3-kinase/Akt signalling was demonstrated in Rat-1 fibroblasts and PC12 cells [20]. In the present study, we explored the roles of signalling molecules in the mitochondria-dependent apoptotic death of PC12 cells treated with 25-OH-chol.

Materials and methods

Cell culture and treatment of 25-OH-chol

PC12 cells, purchased from the American Type Culture Collection (ATCC, Manassas, VA), were maintained at 37°C in DMEM containing 10% horse serum, 5% foetal bovine serum and antibiotics in a humidified 5% CO_2 incubator. Cells were plated at 1×10^5 cells per well in a 48-well culture plate and 2 days later cells were used for the experiments. SB415286, SB216763 and SP600125 (Calbiochem, San Diego, CA) were added to the cell cultures and then treatment of 2 μM 25-hydroxycholesterol (Sigma Chemical Co, St. Louis, MO) for 48 h.

Cell viability and morphological observation

Cell viability was determined by the conventional MTT (Sigma Chemical Co, St. Louis, MO) reduction assay. Cells were incubated with 500 μg/ml MTT for 2 h at 37°C. The dark blue formazan crystals formed in intact cells were solubilized with DMSO and absorbance at 595 nm was measured with a microplate reader (Bio-Tec Instruments, Winooski, VT). Results were expressed as the percentage change of MTT reduction.

The extent of cytotoxicity of 25-OH-chol was also determined by lactate dehydrogenase (LDH) activity released into culture media using the CytoTox96 non-radioactive assay (Promega, Madison, WI) and quantitated by measuring wavelength absorbance at 490 nm. Total LDH amount corresponding to complete PC12 cells damage/death was measured in a sister culture treated with 0.1% Triton X-100 for 30 min at 37°C.

PC 12 cell injury and death was also assessed by morphological examination using phase-contrast microscopy or fluorescence microscopy. For fluorescence microscopy, cells were fixed for 15 min with 4% paraformaldehyde at room temperature. Cells were then washed twice with PBS and stained with propidum iodide (1 μg/ml) or DAPI (0.1 μg/ml) for 30 min at room temperature. After three washes with PBS, cells were observed under a fluorescence microscope (Olympus Co, Japan).

Western blot

For Western blot analysis, cells were harvested by centrifugation at $750 \times g$ for 5 min at 4°C. The cell pellets were resuspended in 1 ml of 10 mM HEPES-KOH, pH 7.4, 1% Triton X-100, 7.5 mM $MgCl_2$, 2 mM EGTA 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride and protease inhibitor cocktail and then homogenized. The homogenates were kept at −70°C for 10 min and then thawed at room temperature. Whole cell lysates were prepared in lysis buffer (10 mM Tris pH 8, 140 mM NaCl, 1% Triton, 0.5% SDS and protease inhibitors) and cleared from cellular debris by centrifugation at $10\,000 \times g$ for 15 min at 4°C. Protein samples (30 μg for each) were separated by 12% SDS/polyacrylamide gel (SDS–PAGE) and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 3% non-fat milk in TBST solution (10 mM Tris-HCl containing 150 mM NaCl and 0.1% Tween 20) at room temperature for 1 h and then incubated overnight at 4°C with primary antibodies. Membranes were washed three times for 5 min each with 10 ml of TBST. After washing with TBST, horse radish peroxidase (HRP)-conjugated secondary antibodies (1:3000 dilution in TBST; Santa Cruz Biotechnology, Santa Cruz, CA) were applied and the blots developed by the Enhanced Chemiluminescence (ECL) detection system (Lab Frontier, Seoul, Korea). The following primary antibodies were used: anti-caspase -3, -9 antibody (1:1000), α-spectrin (1;10 000), PARP (1:3000), cytochrome C antibody (1:1000) and anti-β-actin antibody (1:3000) obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-JNK antibody (1:1000), anti-phospho-Akt (1:1000) and anti-phospho-GSK antibody (1:1000) purchased from Cell Signaling Technology (Beverly, MA).

Measurement of caspase 3, 8 and 9 protease activity

PC12 were harvested and lysed in a lysis buffer containing 10 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, 2 mM EGTA, 2 mM EDTA, 1 mM PMSF and protease inhibitor cocktail. Lysates were centrifuged at 12 000 g for 10 min at 4°C and the supernatants were collected. The supernatants (10 μl) were incubated in a 96-well plate with 90 μl of assay buffer (10 mM Hepes, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 10% sucrose) containing 30 μM caspase substrate (caspase-3

substrate Ac-DEVD-AMC, caspase-8 substrate Ac-IETD-AFC or caspase-9 substrate Ac-LEHD-AFC) (Calbiochem, San Diego, CA). AMC from Ac-DEVD-AMC was measured on a microplate fluorescence reader (Bio-Tec Instruments, Winooski, VT) with an excitation wavelength of 360 nm and an emission wavelength of 480 nm and AFC from Ac-IETD-AFC or Ac-LEHD-AFC was measured on a microplate fluorescence reader (Bio-Tec Instruments, Winooski, VT) with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Isolation of cytosol and mitochondrial fractions

Cells (20×10^5) were plated in a 100 mm dish. Cells were harvested by centrifugation at $750 \times g$ for 10 min at 4°C. The cell pellets were resuspended in 1 ml of 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 250 mM sucrose. Cells were then broken open with six passages through a 26-gauge needle applied to a 1-ml syringe. The homogenate was centrifuged at $750 \times g$ for 10 min at 4°C to remove nuclei and unbroken cells. The supernatant was transferred to a high speed centrifuge tube and centrifuged $10\,000 \times g$ for 15 min at 4°C. The resulting mitochondrial pellet was lysed in 50 μl of 20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, protease inhibitor cocktail and 250 mM sucrose and then incubated on ice for 15 min. The supernatant from the $10\,000 \times g$ spin was centrifuged at $100\,000 \times g$ (60 min at 4°C) and the supernatant was used for preparation of cytosol. The cytosolic fraction was concentrated through a Microcon YM-10 Centrifugal Filter Device (Millipore, Bedford, MA). Protein content of the fractions was determined by the bicinchoninic acid assay (Sigma Chemical Co, St. Louis, MO).

Measurement of intracellular ROS levels

The fluorescent dye 2′, 7′-dichlorodihydrofluorescenin diacetate (H_2DCFDA, Molecular Probes, Invitrogen, Carlsbad, CA) was used to determine the intracellular ROS level. Cells (2×10^5) were plated in 24-well culture plate and treated with 25-hydroxycholesterol for 24 h. To measure the ROS production, cells were loaded with 20 μM H_2DCFDA for 30 min at room temperature. Several fields per sample were selected randomly and then fluorescence images were captured with a microscope (Olympus Co, Japan). We determined the fluorescence intensity of H_2DCFDA by using an image analysis program (Tomoro scope Eye 3.5, Techsan Corporation, Seoul, Korea).

Measurement mitochondria permeability transition pore (MPTP) opening in cells

Mitochondrial membrane potential was measured utilizing the cationic and lipophilic dye TMRE, which accumulates in the mitochondrial matrix. Changes in mitochondrial membrane potential are indicated by a reduction in the TMRE-induced red fluorescence intensity. Following the end of exposure to 25-OH-chol, cells were incubated with TMRE (20 nM) for 20 min at room temperature. Several fields per sample were selected randomly and then fluorescence images were captured at excitation 568 nm and emission 590 nm wavelengths with a microscope (Olympus Co, Japan). The fluorescence intensity of TMRE was determined by using an image analysis program (Tomoro scope Eye 3.5, Techsan Corporation, Seoul, Korea) and corrected for autofluorescence.

Statistical analysis

Data were expressed as means ± SEM. All experiments were performed in triplicate and repeated at least three times. Data were analysed by ANOVA followed by Dunnett's multiple comparison method unless otherwise stated.

Results

25-OH-chol induces apoptosis in PC12 cells

Measurements of MTT reduction and LDH release showed that treatment of 25-OH-chol for 48 h reduced the viability of PC12 cells in concentration-dependent manners (Figure 1A and B). PC12 cells treated with 25-OH-chol displayed multiple features of apoptosis, including nuclei condensation and fragmentation (Figure 1C and D, respectively).

25-OH-chol increases the activity of caspase-9 and -3

One of the major apoptotic cell death signalling pathways is the activation of caspases via release of cytochrome c from injured mitochondria. Thus, we examined whether 25-OH-chol released cytochrome c from mitochondria and altered the activities of caspases. Western blot demonstrated that treatment with 25-OH-chol decreased the level of mitochondrial cytochrome c but increased that of cytosolic cytochrome c, indicating that cytochrome c was released from mitochondria into cytosol (Figure 2A). Western blot further showed that the amounts of active forms of caspase-3 and -9 were increased in 25-OH-chol-treated cells (Figure 2A). Using fluorogenic peptide substrates (Ac-LEHD-AFC for caspase 9 and Ac-DEVD-AMC for caspase 3), we found that 25-OH-chol increased the activities of both caspase-9 and -3 (Figure 2B and C). However, 25-OH-chol did not alter the activity of caspase-8 (data not shown).

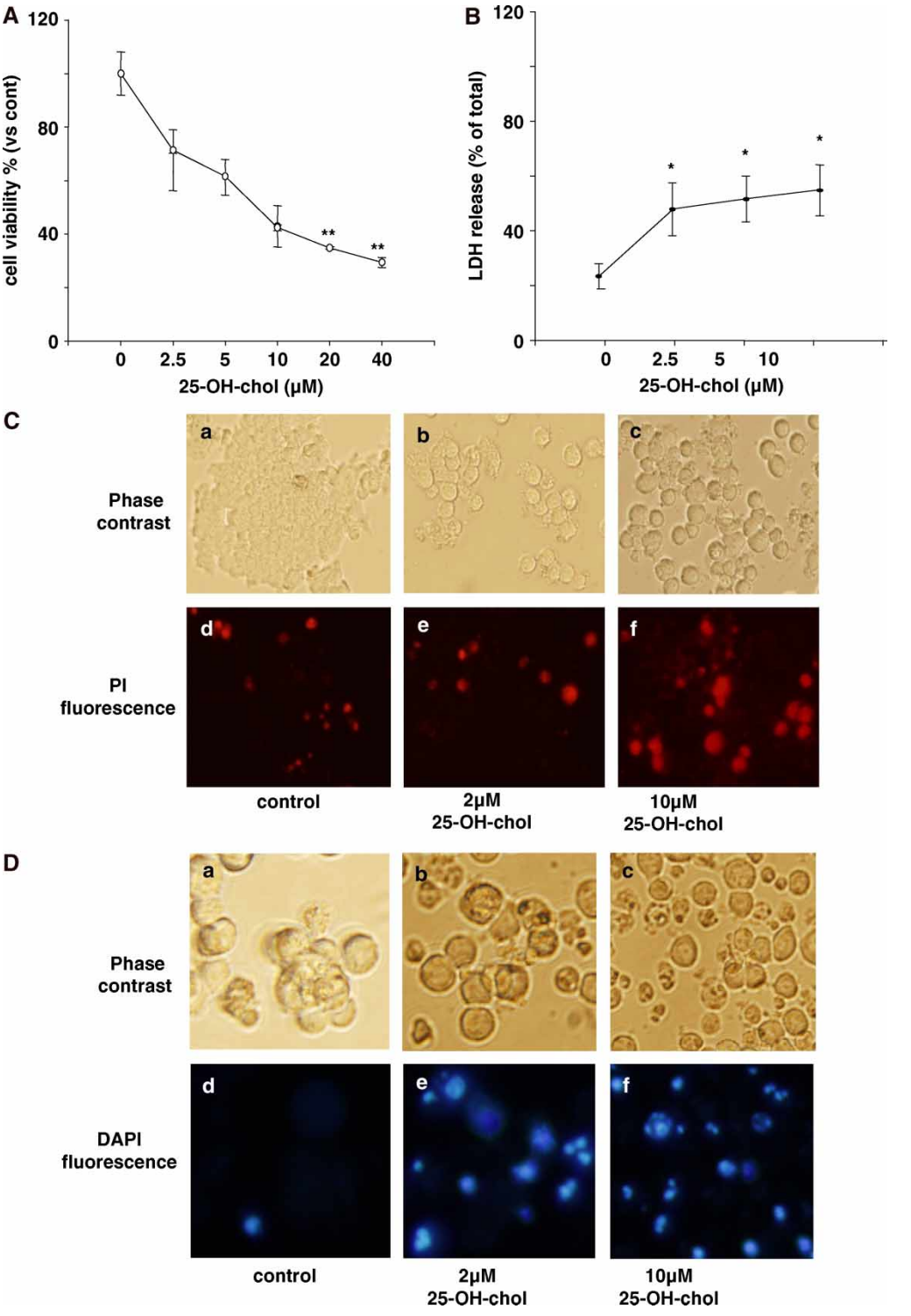

Figure 1. 25-OH-chol induces apoptotic cell death in PC12 cells. (A and B) PC12 cells were treated with or without 25-OH-chol at various concentrations. After 48 h, cell viability was determined by MTT assay (A) or LDH release (B). Data are presented as mean ± SEM ($n = 3$) $^{\star}p < 0.001$, compared with the group not treated with 25-OH-chol. (C and D) Cells were treated with 2 or 10 μM 25-OH-chol for 48 h in serum-free medium and then stained with propidium iodide (C) or DAPI (D) ($n = 4$).

The cleavage of poly (ADP-ribose) polymerase (PARP) and α-spectrin by caspase-3 is a common and physiologically significant step in the execution phase of apoptosis [21]. Expectedly, the activation of capsase 3 by 25-OH-chol was further confirmed by the cleavage of PARP and α-spectrin (Figure 2D).

25-OH-chol induces ROS production and MPTP opening

ROS have been suggested as possible mediators of the apoptosis induced by oxysterols. Using a fluorescent probe H_2DCFDA, we found that 25-OH-chol increased the intensity of fluorescence after 24 h exposure (Figure 3A and C). Mitochondrial

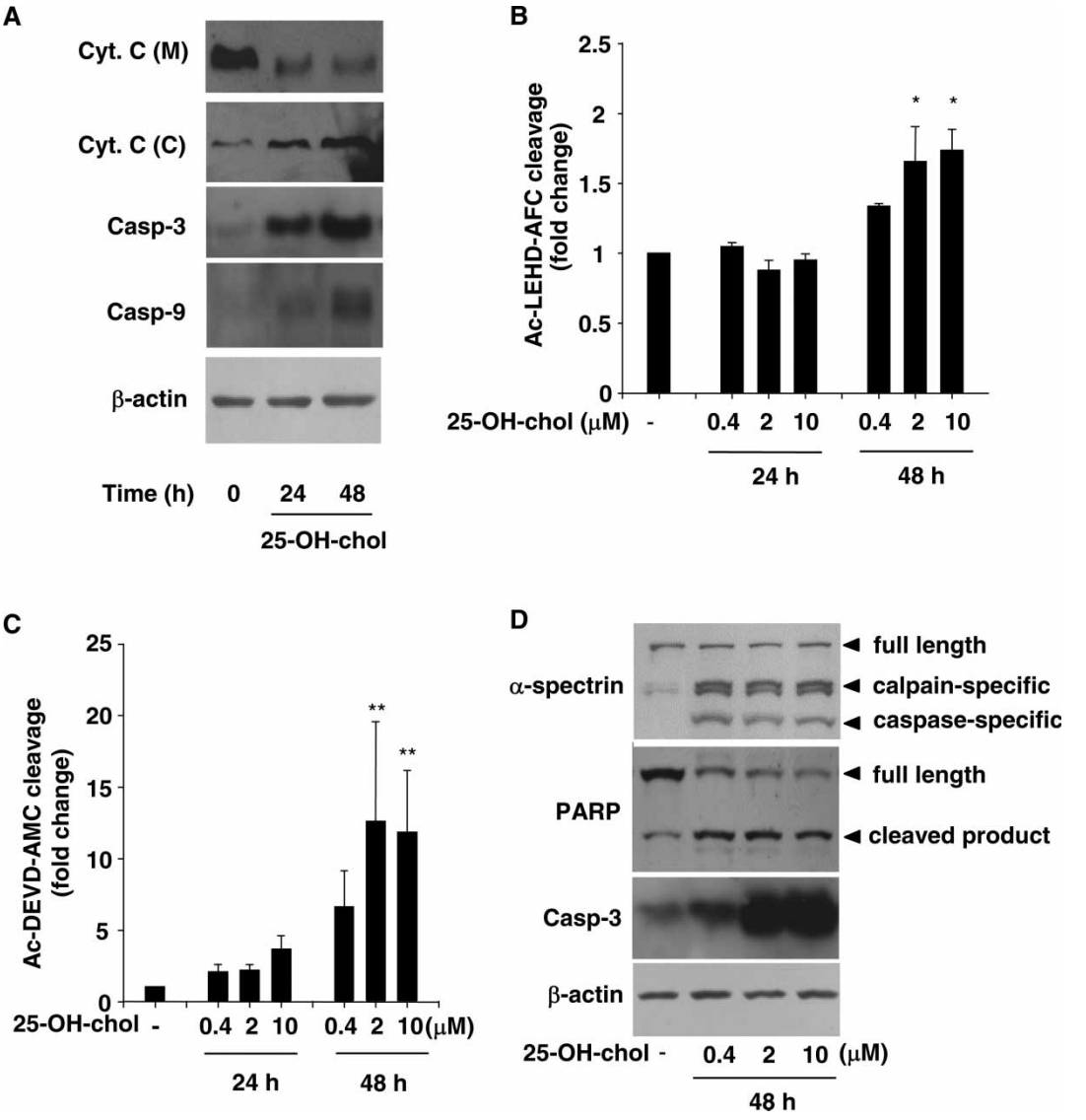

Figure 2. 25-OH-chol induces cytochrome c release and caspase activation. (A) Cells were treated with 2 μM 25-OH-chol for 24 and 48 h and then harvested by centrifugation at 750 × *g* for 10 min at 4°C. Mitochondrial and cytosolic extracts were prepared as described in Materials and methods. (B and C) Cells were treated with 0.4, 2 and 10 μM 25-OH-chol for 24 and 48 h. Cells were harvested, lysed and then the activity of caspase-9 (B) or -3 (C) were determined by LEHD-AFC or DEVD-AMC cleavage assay. Data are presented as mean ± SEM from three separate experiments. * $p < 0.01$; ** $p < 0.05$, compared with untreated group. (D) Cells were treated with 0.4, 2 and 10 μM 25-OH-chol for 48 h. Cell lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. Data for (A) and (D) are representative of at least six independent experiments.

dysfunction is characterized by the increase of MPTP opening. Thus, mitochondrial membrane permeability was further assessed by measuring the fluorescence ratio of TMRE. At 24 h after addition of 10 μM 25-OH-chol, TMRE fluorescence was decreased in PC 12 cells, implying that MPTP was opened (Figure 3B and D).

25-OH-chol increases phosphorylation of JNK

To investigate the signalling pathways mediating 25-OH-chol-induced apoptosis, we first examined changes in the activity of MAPKs. We found that 25-OH-chol stimulated JNK phosphorylation in a concentration-dependent manner (Figure 4A). However, levels of ERK1/2 and p38 phosphorylation were not altered by 25-OH-chol (data not shown). The JNK inhibitor SP600125 attenuated 25-OH-chol-induced elevation of caspase-3 activity, as determined by fluorescent substrates (Figure 4B).

25-OH-chol inhibits the activation of Akt and GSK-3β

The phosphatidylinositol-3-kinase/Akt signalling pathway is one of the signalling systems involved in the cell survival that leads to phosphorylation and resultant inhibition of GSK-3*β* [19,22]. We found that 25-OH-chol decreased the levels of

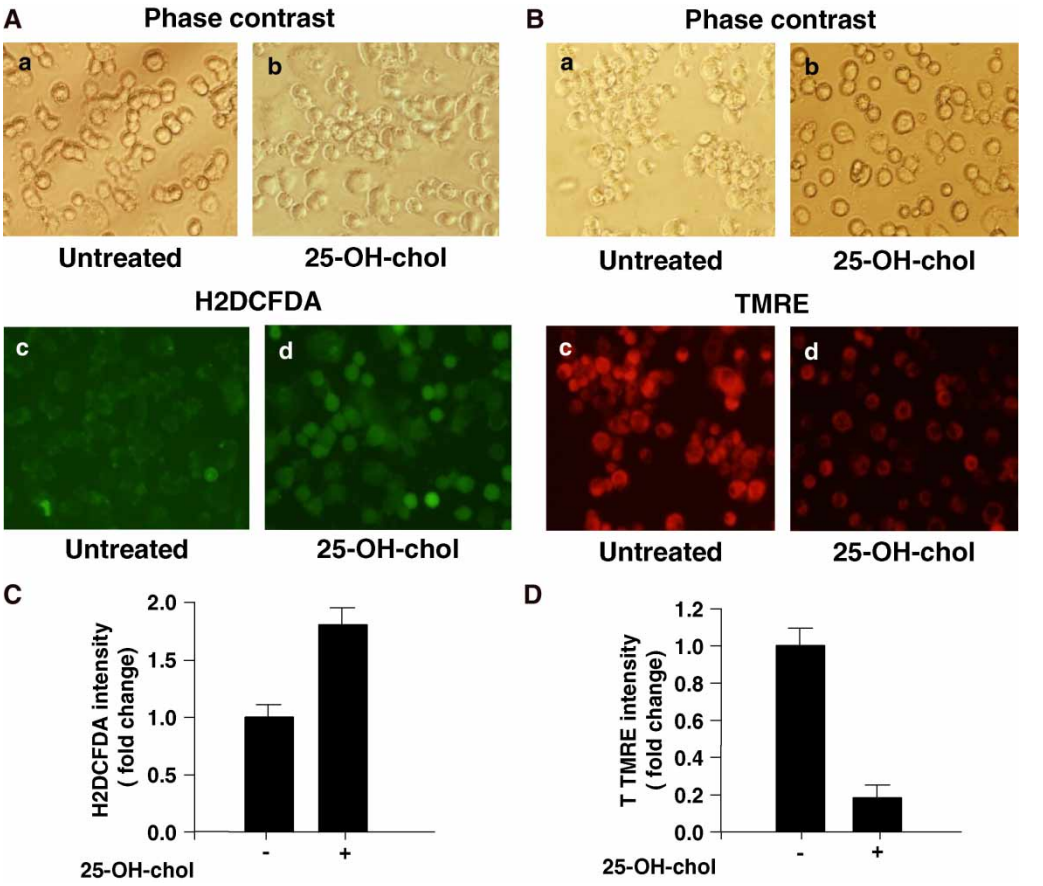

Figure 3. 25-OH-chol induces ROS production and MPTP opening. (A and B) Cells were treated with 10 μM 25-OH-chol for 24 h and then loaded with H_2DCFDA (A) or TMRE (B), as described in Materials and methods. a, b: phase contrast; c, d: H_2DCFDA (A) or TMRE (B) ($n=4$). (C and D) The fluorescence intensity of H_2DCFDA (C) or TMRE (D) were determined and expressed as a fold change.

phosphorylation of Akt and GSK-3β at the residue of Ser-9 (Figure 4A). To determine whether 25-OH-chol-stimulated activities of caspase-9 and -3 were mediated by the activation of GSK-3β, we pre-treated cells with GSK-3β inhibitors (10 μM SB216763 or SB415286) for 1 h and then treated with 25-OH-chol for 48 h. Inhibition of GSK-3β significantly attenuated the activation of caspase-9 and -3 by 25-OH-chol (Figure 5A and B). Western blot analysis also showed that only the caspase-specific cleavage of α-spectrin shown as 120 Kd was markedly blocked by GSK inhibitors (Figure 5C). Thus, calpain-dependent cleavage of α-spectrin shown as 140 and 150 Kd was not blocked by GSK inhibitors (Figure 5C). To study the relationship between GSK and JNK in 25-OH-chol cytotoxicity, cells were pre-treated with the JNK inhibitor SP600125 for 1 h and then stimulated with 2 μM 25-OH-chol for 48 h. We found that the GSK inhibitor SB216763 or SB415286 significantly attenuated the JNK phosphorylation stimulated by 25-OH-chol (Figure 5C). However, the JNK inhibitor SP600125 had little effect on GSK activation (Figure 5D). Inhibition of either GSK or JNK prevented the release of cytochrome c from mitochondria (Figure 5C and D). The present results indicate that GSK-3β induces 25-OH-chol-evoked apoptosis via the JNK/cytochrome c/caspase pathway in PC12 cells.

Discussion

In the present study, we found that 25-OH-chol induced cell death via damage of mitochondrial function. The mitochondrial dysfunction caused by 25-OH-chol was deduced from the results of the MTT test and the opening of MPTP. 25-OH-chol-evoked injury of mitochondria resulted in the release of cytochrome c and subsequent activation of caspase-9 and -3. Prior to mitochondrial damage, 25-OH-chol decreased AKT activity and consequently increased GSK-3β activity. Activation of GSK-3β increased the activity of JNK, a well-known cell death signalling molecule. A schematic diagram depicting

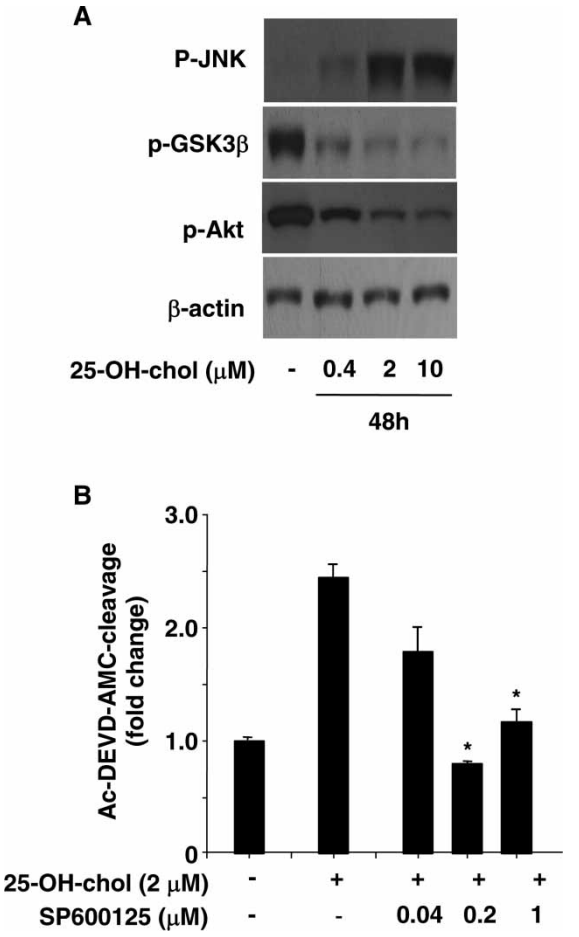

Figure 4. Involvement of JNK in 25-OH-chol-induced apoptosis. (A) Cells were treated with 0.4, 2 and 10 μM 25-OH-chol for 48 h. Western blot analysis was performed with antibodies against p-JNK, p-Akt, p-GSK-3β and β-actin. (B) Cells were pre-treated with or without a JNK inhibitor SP600125 at various concentrations for 1 h and then treated with 2 μM 25-OH-chol for 48 h. Cell lysates were used to determine the activity of caspase -3. Data are presented as mean ± SEM ($n = 3$). * $p < 0.01$, compared with 25-OH-chol-treated group.

the mitochondria-dependent apoptotic pathway for 25-OH-chol is presented in Figure 6.

Our present results are consistent with prior studies showing the activation of mitochondrial injury pathway by oxysterols with its canonical cytochrome c release [7,23]. In the present study, we showed that mitochondrial damage and release of cytochrome c could be caused by two separate pathways: one is the increased production of ROS and the other the decreased activity of pro-survival AKT signalling pathway. The production of ROS was steadily increased in 25-OH-chol-treated PC12 cells. A similar increase of ROS production and mitochondrial dysfunction by oxysterols such as 24-hydroxycholesterol, 25-hydroxycholesterol and 7-ketocholesterol was recently reported in primary porcine retinal pigment epithelail cells [24]. Despite the relatively low production of ROS in oxysterol-treated cells, the cytoprotective effect of various antioxidants such as MnTBAP and N-acetyl cysteine has demonstrated the involvement of ROS in cytotoxicity [25].

Caspase-3 has been shown as one of the key proteins of the apoptotic pathways. This protease is involved in the execution of apoptosis, catalysing the specific cleavage of proteins essential for cell survival [21]. Poly ADP-ribose polymerase (PARP) is a key enzyme involved in genome surveillance and DNA repair [26]. Spectrin is a universally expressed membrane-associated cytoskeletal protein consisting of alpha- and beta-sub-units. α-Spectrin is one of the primary targets cleaved by caspases during apoptosis [27]. In the present study, activation of caspase-3 was accompanied with PARP activation and α-spectrin cleavage in PC12 cells treated with 25-OH-chol.

JNK represents a group of mitogen-activated protein kinases (MAPKs), which is activated when cells are exposed to environmental stresses [28]. Previously, targeted gene disruption studies have established that the c-Jun NH_2-terminal kinase (JNK) is required for the stress-induced release of mitochondrial cytochrome *c* and apoptosis [28]. The Bax sub-family of Bcl-2-related proteins is known to be essential for JNK-dependent apoptosis [28]. Our results revealed that phosphorylation of JNK occurred in the cytoplasm of 25-OH-chol-treated PC12 cells.

Down-regulation of the pro-survival protein kinase AKT (protein kinase B) activity may be a crucial event in the induction of apoptosis by 25-OH-chol. In one way, the activity of AKT can be regulated by phosphorylation. Consistently, in the present study AKT is dephosphorylated after treatment with 25-OH-chol. Other reports have also revealed that AKT degradation might play a role in apoptotic signal transduction pathways. For example, in the murine macrophage-like cell line P388D1, 25-OH-chol induced the degradation of AKT [29]. Furthermore, H_2O_2-induced apoptosis is accompanied by degradation of AKT [30].

AKT has been associated with phosphorylation and inactivation of GSK-3 [19]. GSK-3, especially the beta form of GSK (GSK-3β), plays crucial roles in ROS-induced neuronal cell death [31]. GSK-3β modulates Bax expression and function at the transcriptional and post-translational levels, respectively, to promote mitochondrial apoptosis [32]. Previously, it was reported that GSK-3β physically associates with and activates MEKK1, thereby stimulating the JNK/SAPK pathway [33]. This report might be consistent with the findings that PI3-kinase and AKT act as negative regulators of the JNK/SAPK cascade [34,35]. Treatment with 25-OH-chol increased GSK-3β activity in PC12 cells. The apoptotic death of PC12 cells was attenuated by GSK-3β inhibitors SB216763 and SB415286. However,

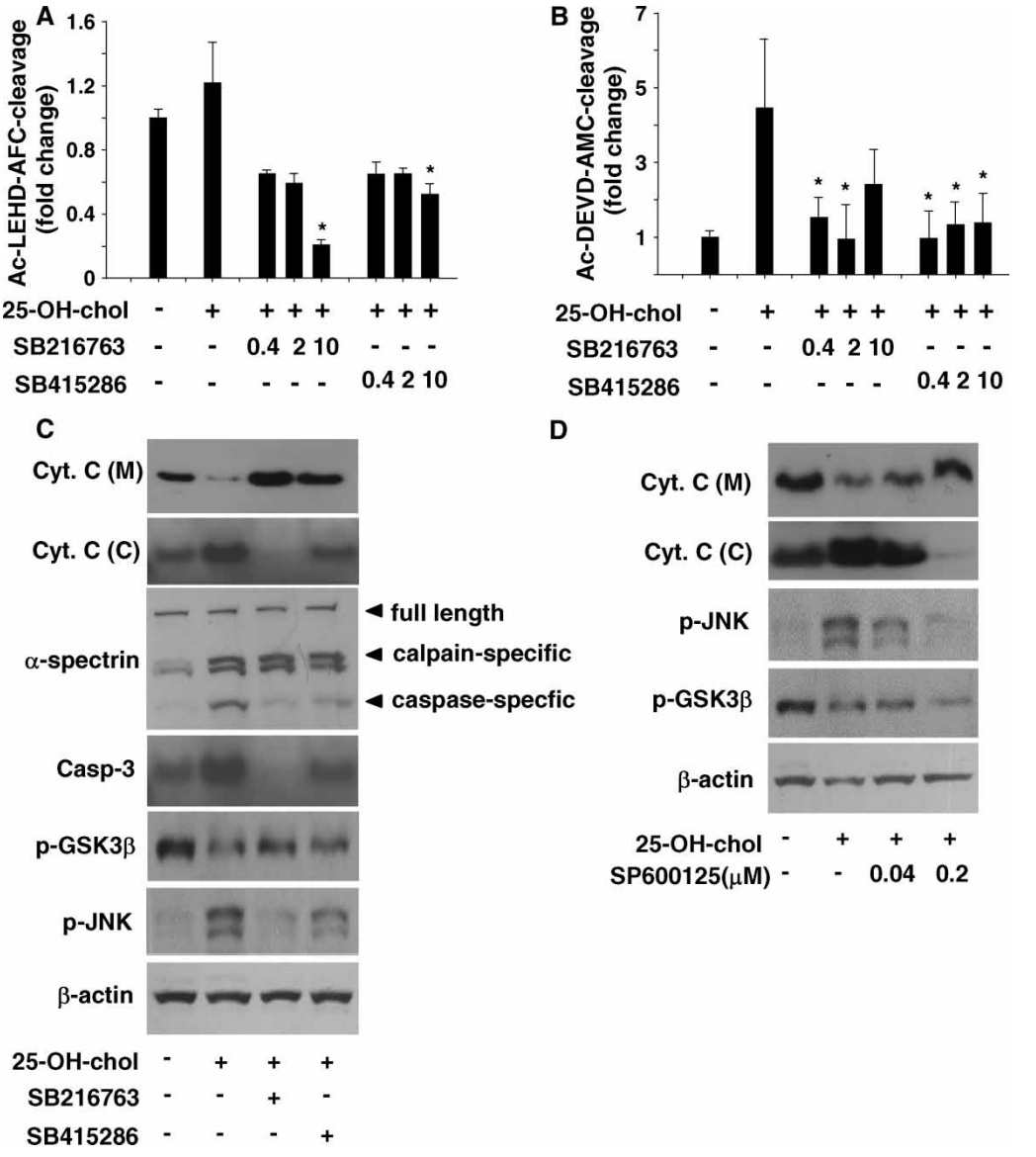

Figure 5. Inhibition of GSK-3β attenuates 25-OH-chol-induced cell death. (A and B) Cells were pre-treated with GSK inhibitors SB216763 (10 μM) or SB415286 (10 μM) for 1 h and then treated with 2 μM 25-Oh-chol for 48 h. The activity of caspase-9 or -3 was determined in cell lysates by using LEHD-AMC (A) or DEVD-AMC (B) cleavage assay. Data are presented as mean ± SEM ($n = 3$). * $p < 0.01$, compared with 25-OH-chol-treated group. (C and D) Protein samples (30 μg/lane) were separated by SDS-PAGE and subjected to immunoblotting with antibodies specific to cytochrome c, caspase-3, α-spectrin, p-GSK-3β, p-JNK and β-actin (C) or those to cytochrome c, p-GSK-3β, p-JNK and β-actin (D). Data are representative of at least four independent experiments.

the JNK inhibitor SP600125 did not significantly inhibit the phosphorylation of GSK-3β stimulated by 25-OH-chol. It implies that GSK-3β directly activates JNK. However, the reverse may not be true. We also further demonstrated that 25-OH-chol-induced activation of GSK/JNK pathway injured mitochondria, resulting in the release of cytochrome c and subsequent activation of caspase pathway.

In conclusion, our data suggest that 25-OH-chol induced PC12 cell apoptosis through the mitochondrial pathway initiated by the generation of reactive oxygen species and the apoptosis-inducing activation of GSK-3β-dependent JNK. In general, cholesterols are transported into mitochondria and oxidized to oxysterols. Thus, pharmacological intervention of cholesterol transport into mitochondria may provide a good therapeutic strategy to reduce the extent or progress of cholesterol-associated diseases.

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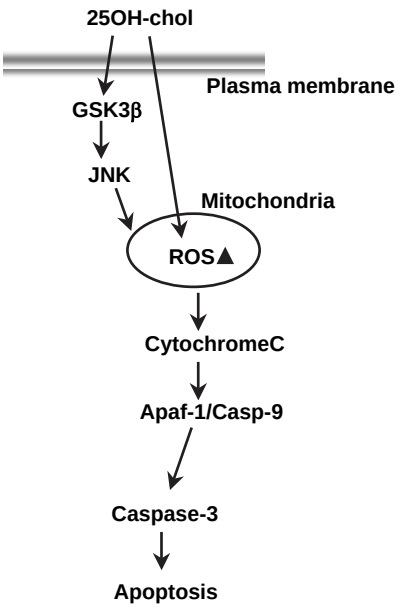

Figure 6. Schematic diagram showing the mitochondria-dependent apoptotic pathway for 25-OH-chol. Activation of GSK/JNK pathway by 25-OH-chol injures mitochondria, resulting in the release of cytochrome c and subsequent activation of caspase pathway.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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